Correlation between folate and vitamin B$_{12}$ and markers of DNA stability in healthy men: preliminary results

Mirta Milić$^{1,2}$, Ružica Rozgaj$^1$, Vilena Kašuba$^1$, Višnja Oreščanin$^2$, Melita Balija$^3$ and Irena Jukić$^3$

$^1$Mutagenesis Unit, Institute for Medical Research and Occupational Health, Zagreb, Croatia; $^2$Laboratory for Radioecology, Ruđer Bošković Institute, Zagreb, Croatia; $^3$Croatian Institute for Transfusion Medicine, Zagreb, Croatia

The aim of this study was to find correlations between folate and vitamin B$_{12}$ on baseline damage in white blood cells and their association with smoking, alcohol consumption and ageing. Thirty-six healthy vitamin non-deficient male subjects were selected in a randomized study. Comet assay (SCGE) and micronucleus (MN) assay were used as biomarkers of DNA damage. The amount of DNA damage was correlated with vitamin B$_{12}$ and folic acid concentration. Positive, but non-significant correlation (canonical $R = 0.61$; $\chi^2 = 28.97$; $P = 0.253$) was found between micronucleus (MN) frequency or comet assay parameters (SCGE) and five covariates (age, smoking, alcohol consumption, vitamin B$_{12}$ and folate blood serum concentration). The highest MN frequency was observed in the group with the lowest vitamin B$_{12}$ concentration ($F = 3.59$; $P = 0.024$). The SCGE assay failed to show significant correlation with vitamin B$_{12}$ or folic acid concentration. Concentration of vitamin B$_{12}$ was significantly correlated with incidence of micronuclei. Our results present background data that could be valuable for future genotoxicological monitoring.

Keywords: folic acid, vitamin B$_{12}$, micronucleus, comet assay, healthy men, DNA damage

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INTRODUCTION

Dietary habits play an important role in maintaining genome stability. An association between micronutrient intake (such as folate and vitamin B$_{12}$) and cancer incidence has been observed (Fenech, 2003a; Ames, 2001). Reduced thymidylate (TMP) synthesis has been associated with folate-sensitive fragile sites (Sutherland, 1988; Zingg & Jones, 1997), development of malignancies (Popescu, 2003) and with increased uracil misincorporation (Eto & Krumdieck, 1986; Fenech, 2001). Uracil removal can generate point mutations, lead to single and double DNA strand breaks, chromosome breakage, especially chromosome 21 aneuploidy (Beetstra et al., 2005), micronucleus formation (Blount & Ames, 1995; Duthie, 1999) and malignant transformation (Basten et al., 2006). Inadequate folate intake is associated with increased risk of neural tube defects, Down syndrome, cardiovascular disease, Alzheimer’s disease and various cancers (Kažimirova et al., 2006). An association between folate status and micronucleus frequency was also found in folate non-deficient individuals (Fenech et al., 1998).

Vitamin B$_{12}$ is required for the synthesis of S-adenosylmethionine (SAM), a common methyl donor required for the maintenance of methylation patterns in DNA that determine gene expression and DNA conformation (Zingg & Jones, 1997). Low concentrations of SAM lead to DNA hypomethylation, associated with abnormal gene expression (Fenech, 2003) and chromosomal segregation (Fenech, 2003) in specific regions such as the centromere, chromosomes 1, 9 and 16 (Tuck-Muller et al., 2000), and fragile sites (Sutherland, 1988; Zingg & Jones, 1997). Very low intake of vitamin B$_{12}$ leads to anaemia and nervous system damage (Kažimirova et al., 2006). Folate and/or vitamin B$_{12}$ deficiency can cause chromosome damage and micronucleus formation in humans in vivo (Kažimirova et al., 2006) and in human cells in vitro (Fenech, 2001).

Genome stability is under the influence of exogenous factors (alcohol intake, smoking). Alcoholism is associated with significantly reduced levels of folate, vitamin B$_{12}$ and B$_{6}$ in humans (Glória et al., 1998). Excessive urinary folate excretion associated with acute or chronic ethanol ingestion predisposes to the development of folate deficiency (Hamid & Kaur, 2006). Smoking and ageing can also increase micronucleus frequency (Zjiho et al., 2003; Gabriel et al., 2006).

The most widely used method for DNA damage assessment in human cells is the micronucleus (MN) assay based on counting MN in once-divided binucleated cultured cells after the cytokinesis block with cytochalasin B. MN originate mainly from chromosome breaks or whole chromosomes that have failed to engage with the mitotic spindle when the cell divides (Fenech et al., 2003).

Another technique, alkaline comet assay (single-cell gel electrophoresis, SCGE) is an easy-to-use, quick and very sensitive method for detecting primary DNA strand breaks, that is, direct DNA damage within single cells (Tice et al., 2000; Collins, 2004) and can be applied to proliferating and non-proliferating cells (Kassie et al., 2000) to determine DNA damage as a result of endogenous factors, lifestyle (Morillas et al., 2002; Hoffman & Speit, 2005), and occupational (Palus et al., 1999; Zhu et al., 1999) and environmental exposure (Srám et al., 1998; Valverde et al., 1999).

$^1$e-mail: mmilic@imi.hr

Abbreviations: BRT, total number of nucleoplasmic bridges; BF, total number of nuclear buds; CBMN, cytochalasin B blocked micronucleus test; FA, folic acid; MN, micronucleus; NDI, nuclear division index; SAM, S-adenosylmethionine; SCGE, single-cell gel electrophoresis; TI, tail intensity; TL, tail length; TM, tail moment
The accuracy of any risk assessment, especially low-dose exposures, depends on both the resolution of the method, and the baseline data obtained in well-selected controls. The use of several independent endpoints improves the resolution and provides a more accurate risk assessment (Major et al., 1998).

This study provides data on two biomarkers in peripheral blood samples collected from healthy male subjects randomly selected from the general population of the Republic of Croatia. The aim of the study was to characterise the variability in baseline damage in white blood cells, to investigate association with external and internal factors, and to establish background data useful as control values in future biomonitoring studies.

MATERIALS AND METHODS

Population characteristics. Thirty-six male blood donors (mean age 37.67 ± 9.26, age range: 22–56 years, see Table 1) of mixed social class and occupation (students, teachers, retired, clerks, drivers, waiters, policemen, and unemployed) were informed of the study scope and experimental details, have filled a standardised questionnaire designed to obtain relevant information on the current health status, medical history, and lifestyle, and gave their written consent, submitted and approved by the local Ethics Committee. The questionnaire included data on exposure to possible confounding factors: smoking, alcohol consumption, use of medicines, contraceptives, severe infections, or viral diseases over the past six months, vitamin intake, recent vaccinations, presence of known inherited genetic disorders and chronic diseases, family history of cancer, exposure to diagnostic X-rays, and previous radio- or chemotherapy. Two donors were excluded from the study (one for a recent virosis, and the other who had three diagnostic X-ray examinations during past year).

The enrolled subjects did not differ much in dietary habits and none had a history of occupational exposure to known genotoxic chemicals. Concerning the answers about regular daily alcohol consumption in the questionnaire, and since no one in the group exceeded the normal daily limit (1 dl of wine, 2 dl of beer, 0.3 dl of strong alcoholic drink), we have divided them into two subgroups considering the age, smoking, alcohol, vitamin B12 and FA concentration.

Blood sampling. Peripheral blood (10 ml) was collected under sterile conditions by venipuncture, then 5 ml of heparinised blood was used for SCGE and MN assay. After collection, all blood samples were randomly coded and processed as quickly as possible (usually within 2 h following the blood sampling). Five milliliters of heparinised blood was used for SCGE and MN assay. After collection, all blood samples were randomised and subjected to known genotoxic chemicals. Concerning the answers about regular daily alcohol consumption in the questionnaire, and since no one in the group exceeded the normal daily limit (1 dl of wine, 2 dl of beer, 0.3 dl of strong alcoholic drink), we have divided them into two subgroups considering the age, smoking, alcohol, and vitamin B12 and FA concentration.

RESULTS

Relevant demographic data of the study group are shown in Table 1. Table 2 shows group data...
Micronucleus test

The results of MN assay are shown in Table 2. Total number of MN increased linearly with age. Unexpectedly, the highest MN frequency was in the group with the lowest vitamin B12 concentration (F = 3.59; P = 0.024). The same was not observed for FA concentration, as the second and the fourth group, ranging 5.0–7.7 µg/l and >9 µg/l respectively, showed two times higher mean values of MN when compared with the others. The total number of BT increased with age, alcohol consumption, smoking, decrease in vitamin B12, and increase in FA. BRT values correlated with alcohol consumption and smoking. Unexpectedly, the highest BRT values were observed in the group with the highest FA concentration. NDI (mean value: 2.07 ± 0.31; range: 1.23–2.51) did not show significant variation between the subgroups for any of the grouping variables.

Comet assay

The results of comet assay are shown in Table 2. None of the studied covariates affected TL, TI or TM averages. Smokers had an average tail length of 14.72 ± 1.50 µm and non-smokers, 14.63 ± 1.11 (see Table 2).

As regards vitamin B12, the largest TL was observed in the second group in which vitamin B12 range was 400–500 ng/l, and dropped linearly to the fourth group.

When the FA subgroups were observed, the TL and TM values were the highest in the first group with FA concentration <5 µg/l. The highest TM values were observed in the oldest group.

Coefficient of variation and general regression model

Figure 1. shows that the MN assay parameters had a higher coefficient of variation than the SCGE parameters. The lowest coefficient was found for TL, followed by NDI, while the highest was observed for BRT and BT. Generally, the coefficient of variation decreased with age and the concentration of vitamin B12.

In order to predict which covariate or group of covariates had the highest influence on each dependent variable, we used the general linear model. The results are presented as Pareto charts for each dependent variable (Fig. 2). From the z values it is obvious that of the five grouping variables, vitamin B12 concentration had the highest (statistically significant) influence on the total number of MN. Age and the combination of alcohol and smoking were risk factors as well. Vitamin B12 concentration also had the highest influence on the BT, while the FA concentration was the most important risk factor for the BRT and NDI. It seems that the combination of alcohol, smoking, and low vitamin B12 concentration had the greatest effect on the TI and TM.
Canonical correlation analyses showed a positive insignificant correlation (canonical R = 0.61; $\chi^2 = 28.97; P = 0.253$) between the MN and SCGE parameters and the five covariates (age, smoking, alcohol consumption, vitamin B$_{12}$ and FA) in the healthy males.

Vitamin B$_{12}$ concentration had the highest influence on the MN and BT and on TI and TM. BRT and the NDI depended on the concentration of FA, while the TL mostly depended on age.

DISCUSSION

In this study, we used two different biomarkers, one of exposure (SCGE assay), and the other of effect (MN assay) to evaluate background DNA damage in white blood cells of healthy blood donors randomly selected from the general Croatian population. The main aim was to investigate the association between the two biomarkers and several external and internal factors, as well as the relationship between those factors.

The study shows a positive association between an increased number of MN and lower plasma vitamin B$_{12}$ concentrations in healthy population, implying an involvement of vitamin B$_{12}$ levels in maintaining DNA integrity, not just in vitamin B$_{12}$ deficient population, but also in a healthy one. There is a small number of case studies that link vitamin B$_{12}$ deficiency with increased levels of chromosome damage (Fenech, 2001). Glória et al. (1997) and Fenech et al. (1997) have shown significant negative correlation between the serum vitamin B$_{12}$ baseline levels and MN frequency. A study of MacGregor et al. (1997) has shown that elevated MN index is strongly associated only with low levels of serum folate (less than 4 ng/l) and low levels of plasma vitamin B$_{12}$ (less than 200 pg/l). None of our study subjects had so drastically low values of either, and our results could not be compared.

Our study has shown that the impact of vitamin B$_{12}$ and folate can be also seen in healthy, non-deficient population. Our results had comparable mean values of FA and B$_{12}$ to those of the study of Odagiri and Uchida (1998) on 33 non-folic and non-vitamin B$_{12}$ deficient individuals (FA = 7 ng/l, vitamin B$_{12}$ = 544 pg/l). However, they are a little higher than in the case control study on the healthy Italian population by Cafolla et al. (2000) or in the study by Zijno (2003), suggesting that the Croatian diet may be somewhat richer in folate.

Although there is evidence that recommended daily intake of micronutrients is suboptimal for the protection from genotoxic damage, our results, and also the results of Zijno et al. (2003), indicate that the relationship between genetic integrity and FA and vitamin B$_{12}$ status could be more complex and influenced by other factors such as tobacco smoke and alcohol consumption.

Data on possible age-related increase in primary DNA damage in white blood cells are contradictory (Singh et al., 1991; Mendoza-Núñez et al., 2001). According to some authors, age appears to have no significant effect on the mean basal level of DNA damage (Wojewódzka et al., 1999; Zhu et al., 1999), as was also observed in our study.

Numerous studies have shown an increase in MN frequency with age, confirming MN as a sensitive marker of ageing (Zijno et al., 2003; Kazimirova et al., 2006). Fenech and Rinaldi (1994) found that age was the most important factor influencing the variance of MN in
women and men, while micronutrient level had no apparent significant effects on genetic damage. While our results suggest that vitamin B$_{12}$ concentration may have more influence than FA, Odagiri and Uchida (1998) found, by adjusting the MN index for age, that FA indeed had a protective effect against DNA damage.

The differences in vitamin B$_{12}$ plasma concentrations between smokers and non-smokers are inconsistent from study to study. Although Gabriel et al. (2006) have shown lower plasma vitamin B$_{12}$ levels in smokers than in non smokers, we found higher vitamin B$_{12}$ concentrations and lower MN frequency and FA level in smokers. The explanation for this finding can be that B$_{12}$, as a methyl donor, has camouflaged the real effect. On the other hand, smokers had higher frequency of BT and BRT, which may be explained by the fact that different mechanisms are involved in the origin of MN and BT. Lindberg et al. (2007) have shown different mechanisms of origin of BT and MN in normal and folate-deprived human binucleate lymphocytes. They have shown an increase of MN and BT in FA-deprived cultures, with terminalacentric fragments.

Zijno et al. (2003) suggested that vitamin B$_{12}$ may interact with genotoxic components of tobacco smoke. Some studies have clearly shown genotoxic effects of tobacco smoke, as well as its modifying effect on genome damage induced by other agents (Palus et al., 1999; Zhu et al., 1999; Hininger et al., 2004; Milić et al., 2008). Other studies however have reported no significant increase in the values of biomarkers evaluated (Srám et al., 1998; Wojewódzka et al., 1999; Hoffman & Speit, 2005). A correlation between micronucleated cells and age, with an increase of 0.2 to 0.3 micronuclei per thousand cells per year, has been reported by Trková et al. (2000). It is believed that this effect is either because of accumulated exposure to mutagens over the lifetime or because of the aging of the spindle apparatus (Burgaz et al., 1999).

Gabriel et al. (2006) showed that when age, sex, alcohol consumption and number of cigarettes were tested to identify the strongest prediction model for folate concentration, smoking was the predominant predictor of MN formation. Regular alcohol consumption was also a significant determinant of MN frequency.

A combination of cytogenetic tests and the SCGE assay in biomonitoring studies makes it possible to compare the relative sensitivities of the two test systems and, therefore, gives us a possible clue about the fraction of the DNA damage detected by the SCGE assay that will lead to fixed mutations (Kassie et al., 2000).
As a biomarker, the comet assay, which detects repairable DNA lesions or alkali-labile sites, reflects current exposure (over the previous few weeks) and actual levels of DNA damage present in white blood cells at the moment of blood sampling. It is known that most of the primary DNA lesions are successfully repaired in a few minutes (4–15 min) (Tice, 1995) to a couple of hours (2–3 h) (Singh et al., 1988). Cytogenetic biomonitoring, on the other hand, detects fixed mutations which persist for at least one mitotic cycle (Kassie et al., 2000) and provides additional information on the DNA damage levels, especially on past exposures. Humans are exposed to a variety of natural or synthetic genotoxic substances which can modify the baseline levels of DNA damage. During the lifetime everyone “accumulates” some level of radiation exposure, mainly due to the background, environmental radiation, diagnostic exposures, or small amounts of radioisotopes ingested with food.

The results obtained in this preliminary study bring a lot of useful background data that could be of value in future genotoxicological monitoring. They also confirm that the SCGE assay and MN assay are sensitive biomarkers and that they need to be further evaluated and standardized for the assessment of DNA damage in human biomonitoring studies and evaluation of accidental exposures.

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